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OXYGEN – A PHYSIOLOGICAL ELECTRON ACCEPTOR IN PHOTOSYNTHESIS?

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It has been known for some time that isolated, illuminated chloroplasts are able to use oxygen as a Hill oxidant, that is, as an electron acceptor in the 'light' reactions of photosynthesis. This photosynthetic reduction of oxygen results in a net consumption of oxygen by the chloroplast suspension, since hydrogen peroxide rather than water is usually the product.[20, 34, 35] Oxygen uptake by illuminated chloroplasts is termed the 'Mehler reaction'. The role of the superoxide anion, O_2^-, in this process is reviewed in detail elsewhere[3], but since the participation of superoxide is of some relevance to a consideration of possible roles of oxygen reduction in photosynthesis, a short and schematic account of the steps involved will now be given.

The first part of the Mehler reaction (and of the Hill reaction) is transfer of reducing equivalents ($2e^-$) along the photosynthetic chain, making them available for reduction of a suitable mediator:

$$H_2O \xrightarrow{\text{PHOTOSYSTEMS}} \tfrac{1}{2}O_2 + 2H^+ + 2e^-$$

In the second part (unique to the Mehler reaction) the mediator (which is 'autoxidisable') passes on reducing equivalents to oxygen:

$$2e^- + O_2 + 2H^+ \xrightarrow{\text{MEDIATOR}} H_2O_2$$

This second reaction is now known to proceed by any one of the three routes depicted in Fig. 1. The identity of the mediator that is involved determines which mechanism of the three applies, though in all cases the overall process may be represented by the sum of the above equations, which is:

$$H_2O + \tfrac{1}{2}O_2 \xrightarrow{2e^-} H_2O_2$$

In its simplest form, photosynthetic oxygen reduction is mediated by a compound ('R' in

Fig. 1a) which transfers electrons singly from the chloroplast to oxygen. Examples of such a compound are the viologen dyes (e.g. methyl viologen), flavin mononucleotide, adrenochrome, anthraquinone and phenazine methosulphate. Superoxide (univalently reduced oxygen) may subsequently dismutate either spontaneously or catalytically:

$$2O_2^- + 2H^+ \longrightarrow O_2 + H_2O_2$$

though it may instead be reduced, e.g. by ascorbate[4, 17] or manganous ions[32], in which case net oxygen uptake is increased. Such effects of ascorbate or Mn^{2+} may be reversed by addition of the enzyme superoxide dismutase, which restores the dismutation reaction and with it the original stoichiometry. This 'single-step' oxygen reduction is important chiefly for electron transport studies *in vitro*[5], while the cytotoxicity of superoxide may be the basis of the herbicidal effects of paraquat.[18]

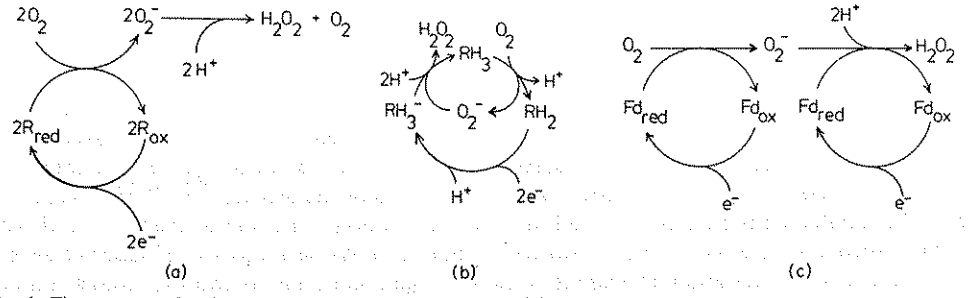

Fig. 1. Three ways of reducing oxygen in photosynthesis. (a) Single-step reduction followed by superoxide dismutation. (b) Two-step reduction by a mediator which has three valence states. (c) Two-step reduction by ferredoxin.

A second mechanism of chloroplast oxygen reduction, first described by Elstner and Heupel[15], is shown in Fig. 1b. Here a mediator such as adrenalin (RH_3^- in Fig. 1b) or dopamine accepts two electrons per molecule from the photosynthetic chain, subsequently donating one electron to oxygen and the other to superoxide. Three valence states of the mediator participate, and the overall reaction is achieved without dismutation of superoxide. Indeed, addition of superoxide dismutase now interrupts the catalytic cycle and hence causes inhibition of both oxygen uptake and photosynthetic electron transport.

The third route of oxygen reduction is the one most likely to be of importance *in vivo*, since it is mediated by an electron carrier, ferredoxin, which is native to photosynthetic systems. As shown in Fig. 1c, this mechanism also involves reduction of both oxygen and superoxide[1], though it differs from that of Fig. 1b in that with ferredoxin only two valence states of the mediator are involved. Addition of superoxide dismutase does not completely inhibit the process, since its presence competitively replaces only one of the ferredoxin-oxidising reactions (superoxide reduction) while the other (reduction of oxygen to superoxide) continues. The relatively high affinity of reduced ferredoxin for superoxide results in a much lower steady-state superoxide concentration than would prevail in the methyl viologen-mediated Mehler reaction, and to this extent the mechanism of reduced ferredoxin's reduction of oxygen to hydrogen peroxide may be regarded as one which which is physiologically 'safe'.

PSEUDOCYCLIC PHOTOPHOSPHORYLATION

'Pseudocyclic' photophosphorylation accompanies non-cyclic electron transport where oxygen is the terminal electron acceptor, and in the presence of an appropriate mediator it is easily observed *in vitro*.[7] It resembles cyclic phosphorylation in that it will occur even where no electron acceptor has been added to an aerobic suspension of chloroplasts, and in the presence of catalase net oxygen exchange does not take place.

It has generally been assumed that ferredoxin will reduce oxygen in chloroplasts only when NADP is absent.[8] This assumption is questioned by the observation[2] that in broken, washed chloroplasts, addition of catalase stimulates net oxygen evolution, an effect which can be understood to result from production of hydrogen peroxide by ferredoxin autoxidation which is occurring even where NADP is the major terminal electron acceptor. The proportion of total electron flux which accounts for reduction of oxygen to hydrogen peroxide in this system can be calculated from the observed rates of net oxygen evolution in the presence and absence of catalase, and at ferredoxin concentrations saturating for electron transport as such, not less than 15% of total electron flow is to oxygen rather than to NADP.[2]

Furthermore, circumstances may arise *in vivo* in which the rate of purely non-cyclic phosphorylation cannot keep pace with demands for ATP made by both CO_2-fixation and biosynthetic processes. Under these circumstances reoxidation of NADPH in the Calvin cycle would be the factor limiting to electron transport, and electrons could consequently be diverted from NADP to oxygen. Even without considering additional biosynthesis, the accepted pathway of CO_2-fixation (in C_3 plants at least) requires ATP and NADPH in the ratio 3ATP:2NADPH, and so an absolute stoichiometry of non-cyclic phosphorylation ($P/2e^-$) of less than 1.5 would create a need for an ancillary reaction producing ATP but not NADPH. Though the $P/2e^-$ ratio of non-cyclic phosphorylation is in dispute[6, 22, 25, 44], even the highest estimates ($P/2e^- = 2$) would not allow non-cyclic phosphorylation by itself to account for the ATP-requirement of CO_2-fixation in C_4 plants (ATP:NADPH = 5:2).[9]

The two chief candidates for the possible ancillary reaction are pseudocyclic and cyclic phosphorylation. Examples of cyclic phosphorylation which appear to depend on the presence of oxygen will be the concern of the next section. Evidence for the occurrence *in vivo* of each of the three main types of photophosphorylation has been discussed by Simonis and Urbach.[42]

Among the various pieces of evidence that pseudocyclic phosphorylation occurs in relatively intact photosynthetic systems, Egneus *et al*.[13] have demonstrated an enhancement by catalase addition of net oxygen evolution in intact, CO_2-fixing spinach chloroplasts. Such chloroplasts were also shown by these investigators to exhibit a light-dependent uptake of ^{18}O-oxygen. Both effects were absent from intact, oxygen-evolving chloroplasts when phosphoglycerate replaced CO_2 as the terminal oxidant; for phosphoglycerate reduction ATP and NADPH are required in equimolar amounts, and so no ancillary ATP synthesis would be required.

Kaiser[30] has also obtained evidence that intact, CO_2-fixing chloroplasts produce hydrogen peroxide, and that added hydrogen peroxide inhibits CO_2-fixation. Kaiser concludes that the hydrogen peroxide which is produced by intact chloroplasts causes inhibition of CO_2-fixation by bringing about depletion of Calvin cycle intermediates, since either catalase or added intermediates (ribose-5-phosphate, fructose-6-phosphate or dihydroxyacetone phosphate) will increase the rate

of CO_2-fixation in a reconstituted chloroplast system, while addition of the same intermediates has little effect when the catalase activity of the chloroplast preparation is high.

Huber and Edwards[26] describe a net oxygen evolution by chloroplasts extracted from mesophyll protoplasts of the C_4 plant *Digitaria sanguinalis*. Oxygen evolution accompanied CO_2-fixation in the presence of pyruvate, and, on addition of cyanide, was replaced by oxygen uptake. The authors regard this effect of cyanide as resulting from an inhibition of catalase with a consequent inhibition of release of oxygen from hydrogen peroxide. Cyanide in their experimental system apparently had no effect on CO_2-fixation itself. They suggest that in C_4 photosynthesis ATP is supplied by both non-cyclic and pseudocyclic phosphorylation.

Raven and Glidewell[38] have shown that light-driven active phosphate transport in whole cells of the green alga *Hydrodictyon africanum* occurs even in the presence of the metabolic inhibitors cyanide and antimycin A, and in the absence of CO_2. In this situation pseudocyclic phosphorylation is likely to be the only remaining source of ATP; oxygen is required for phosphate uptake to take place, and the process is inhibited by DCMU. Glidewell and Raven[19] have also shown that ^{18}O-oxygen uptake accompanies phosphate transport in *Hydrodictyon*.

Using a technique based on fluorescence of scopoletin, Patterson and Myers[36] have demonstrated a light-dependent and DCMU-sensitive production and 'excretion' of hydrogen peroxide by whole cells of a strain of *Anacystis nidulans*. The hydrogen peroxide production was faster under conditions where CO_2-fixation itself is suppressed, though it occurred to a significant extent even when CO_2-fixation was allowed to proceed. They concluded that 'a low potential reductant is produced more rapidly than it can be used in the normal pathway to CO_2 reduction, and, instead, reacts with oxygen'.

More recently, Radmer and Kok[37] have used mass spectrometry to monitor both uptake and evolution of oxygen together with associated CO_2 uptake in suspensions of *Scenedesmus obliquous*, *Chlorella vulgaris* and *Anacystis nidulans*. They found that during a period of 0.5 min immediately following the onset of illumination (corresponding to the lag-phase of CO_2-assimilation), evolution and uptake of oxygen proceeded simultaneously at a rate only 20–30% lower than the steady-state rate of photosynthesis which was ultimately reached (180 μmole/mg chl. per hr). All three exchange reactions were sensitive to the electron transport inhibitor DCMU. Even after the maximal rate of CO_2 uptake had been reached, oxygen uptake accounted for a small proportion of total oxygen exchange, and for a much higher proportion (up to 50%, when net oxygen evolution ceases to be observed) in the presence of inhibitors of CO_2-assimilation such as iodoacetamide, KCN and FCCP. The persistence of oxygen uptake in the presence of uncoupler or inhibitors of the Calvin cycle seems to suggest that a fairly direct photoreduction of oxygen, such as that which occurs via ferredoxin, is taking place in this system. Radmer and Kok[37] conclude that rapid photoreduction of oxygen may be required in these algae to bring about the onset of CO_2-fixation, and that oxygen competes with CO_2 for electrons from the photosynthetic chain in such a way that high rates of electron transport occur under circumstances where CO_2-fixation cannot proceed.

Any photosynthetic reduction of oxygen *in vivo* clearly cannot occur in the same manner as the reaction *in vitro* that is catalysed by the herbicide methyl viologen; here toxicity results from continuous photosynthetic production of superoxide with consequent peroxidation of lipids and damage to membrane integrity. The two-step oxygen reduction which is catalysed by ferre-

doxin is, however, quite acceptable as the basis of a truly physiological pseudocyclic photophosphorylation.

OXYGEN AND CYCLIC PHOTOPHOSPHORYLATION

A number of artificial cofactors are known to support cyclic phosphorylation in isolated chloroplasts under anaerobic conditions. This is also true of ferredoxin, the only known cyclic cofactor which is also a natural chloroplast component. Anaerobic conditions are unlikely, however, to prevail *in vivo*, and so the recent description[6] of an aerobic cyclic phosphorylation which requires ferredoxin at much lower concentrations than does anaerobic cyclic phosphorylation considerably strengthens the claim that cyclic phosphorylation can occur *in situ* in a leaf. According to Arnon and Chain[6] aerobic cyclic phosphorylation requires only the same concentration of ferredoxin (10 μM) as does non-cyclic phosphorylation.

One explanation of this requirement for oxygen of cyclic phosphorylation at low ferredoxin concentrations is that oxygen may act as an oxidant and so regulate ('poise') the system in such a way that an optimum net oxidation–reduction state of the components is achieved. This interpretation is favoured by Arnon and Chain[6], who showed that DCMU inhibits aerobic cyclic phosphorylation in 'photosystem I' light. Such inhibition is likely to result from 'over-oxidation' of the cyclic chain's components, and in the absence of DCMU a slow input of electrons from photosystem II could occur, counteracting the tendency towards over-oxidation which would be caused by ferredoxin's reaction with oxygen.

Inhibition of cyclic electron flow *in vivo* could occur for a similar reason. With a continuous flow of electrons into photosystem I from photosystem II, and in situations where ATP is required and hence pyridine nucleotide is largely reduced, over-reduction would be expected to prevent optimum cyclic electron flow. Oxidation of ferredoxin by oxygen could then provide an 'escape' for electrons and a release of this inhibition. 'Poising' in chloroplasts has been discussed in detail by Grant and Whatley.[21] A PMS-catalysed cyclic phosphorylation which is subject to inhibition by over-reduction under anaerobic conditions has also been described.[45] Poising and hence resumption of phosphorylation could be achieved in this case by addition of oxygen or ferricyanide.

Huber and Edwards[27] have obtained evidence that oxygen, because of its reduction by ferredoxin, causes over-oxidation of the cyclic pathway's components in isolated, intact mesophyll chloroplasts of *Digitaria sanguinalis* under far-red light. Using pyruvate-dependent CO_2-fixation (resulting from the β-carboxylation of C_4 photosynthesis) as a measure of cyclic phosphorylation in this system, they observed an inhibition of CO_2-fixation by oxygen. DCMU also had an inhibitory effect under these conditions, presumably by preventing a supply of electrons from photosystem II, as in the analogous experiment (but with broken spinach chloroplasts incorporating ^{32}P-phosphate) of Arnon and Chain.[6] In white light, however, DCMU stimulated pyruvate-dependent CO_2-fixation in the experiments of Huber and Edwards, an effect which they attributed to a relief by DCMU of over-reduction of the cyclic pathway. If this explanation is correct, and if oxygen does indeed provide an escape route for electrons and hence an alternative antidote to over-reduction, an increase in oxygen concentration should itself stimulate cyclic phosphorylation and associated CO_2-fixation in white light, when both photosystems are active. This oxygen-dependent poising could clearly be of importance in the regulation of any cyclic phosphorylation which occurs *in vivo* in the mesophyll chloroplasts of C_4 plants.

Since cyclic electron transport can result in synthesis of ATP but not of NADPH, cyclic phosphorylation, like pseudocyclic, can provide a solution to the problem, described in the previous section, concerning the stoichiometry of production of ATP and NADPH. In the context of 'poising' it becomes particularly apparent that the operation of neither mode (cyclic or pseudocyclic) of phosphorylation precludes the simultaneous operation of the other.

An alternative explanation for oxygen-enhancement of ferredoxin-mediated cyclic phosphorylation is possible, though as yet it commands no direct experimental support. The basis of this is the number of examples that are known of what can be presumed to be cyclic electron transport in which the O_2/O_2^- couple functions as an electron-carrying component.

In a study of the kinetics of oxidation of plastocyanin by photosystem I, Wood and Bendall[47] found it necessary to add superoxide dismutase to detergent-treated chloroplasts in the presence of methyl viologen in order to suppress reduction of plastocyanin by superoxide. In the absence of superoxide dismutase, a superoxide-mediated cyclic electron transport must presumably have occurred. An analogous reaction has been proposed for *Rhodospirillum rubrum* reaction centres by Boucher and Gingras.[10] Here added horse heart cytochrome c functioned both as an electron donor to the bacterial photosystem and, by virtue of its reduction by superoxide, as an electron acceptor. Takahama and Nishimura[43] taking malondialdehyde formation as a measure of membrane lipid peroxidation in spinach chloroplast fragments, concluded that singlet oxygen (the peroxidising agent) is produced as a result of oxidation of superoxide by components of the inter-photosystem electron transport chain. If this conclusion is correct, and if superoxide is produced when oxygen is reduced by photosystem I, a cyclic system with O_2/O_2^- as a component must have been set up.

Since plastocyanin is now thought to be located near the inner surface of the thylakoid membrane (see Trebst[44]), superoxide, produced at the outer surface of the same membrane (or even in the medium), would not be expected to function as a link for this kind of cyclic electron transport except in detergent-treated or otherwise damaged chloroplasts or chloroplast fragments. This may help to explain an inhibition by ferredoxin of net oxygen uptake in *Euglena* chloroplasts, and in digitonin-treated spinach chloroplast fragments, but not in untreated spinach chloroplasts, as described by Elstner *et al.*[16] Elstner *et al.* attributed the inhibition to a ferredoxin-mediated cyclic electron transfer via cytochrome 552 (in *Euglena* an analogue of plastocyanin).

In vivo, however, the permeability barrier of the thylakoid membrane would prevent a reaction of superoxide with plastocyanin, and, even if it were to occur, such a cyclic pathway would be a wasteful one in the sense that it would not contribute to the transmembrane proton concentration gradient which is held to be the link between electron transport and synthesis of ATP. A contribution to photophosphorylation by a superoxide-mediated cyclic electron transport would occur only if the proton-translocating step involving plastoquinone were to form part of the cycle. The thylakoid would not represent a permeability barrier to such a process, since the site of plastoquinone reduction (and of proton binding) is located, like the site of oxygen reduction by photosystem I, on the outer surface of the membrane.[44, 29] Cytochrome b_{563} is thought to mediate electron transfer from ferredoxin in the cyclic pathway.[41] The possibility that O_2/O_2^- may also represent a link in this chain cannot yet be ruled out on either thermodynamic or kinetic grounds, though experimental support for this idea has yet to emerge.

SUPEROXIDE AND NET TRANSLOCATION OF PROTONS

Following the experiments of Junge and Auslander[29] the net proton-translocating capacity of illuminated thylakoids can be seen to result from the existence of two sites of proton binding at the outer surface of the thylakoid and two sites of proton production at the inner surface. In these experiments absorbance changes of the dye cresol red were used to measure pH changes at the outer surface of the thylakoid. The autoxidisable electron acceptor benzyl viologen was used, and proton-binding at photosystem I would be expected to result from the formation of undissociated hydrogen peroxide which follows dismutation of the superoxide anion.

Schmid[40] has reported that this proton-binding step seems not to occur in chloroplasts which which have been EDTA-treated in order to remove the chloroplast coupling factor. In such chloroplasts Schmid found only a net acidification following a single-turnover flash, whereas in chloroplasts not treated with EDTA an initial alkalinization at the outer surface of the thylakoid is followed by a relatively slow return to the initial pH. Schmid[40] proposed that EDTA-treatment removes an intrinsic chloroplast superoxide dismutase, and thereby 'makes O_2^- a rather stable end product of the electron transport'. The proton uptake associated with the dismutation is thus avoided, and net acidification results. In support of this explanation, addition of superoxide dismutase to EDTA-treated chloroplasts was shown to restore the kinetics of proton uptake which were found in chloroplasts which had not been treated with EDTA.

On a millisecond time-scale it may be not unreasonable to regard superoxide as 'rather stable', though Schmid's results show that the effect of acidification persists for several seconds after illumination. It would be of interest to know if a similar effect could be observed with cofactors which autoxidise by a two-step mechanism.

PHOTORESPIRATION

The reductive pentose phosphate pathway of photosynthetic CO_2-fixation[11] is a process which does not itself require oxygen, even if the ATP which it consumes may in certain circumstances have been manufactured in an oxygen-requiring photophosphorylation. The same is true of the additional steps of CO_2-fixation found in C_4 plants.[24] However, in photorespiration, a phenomenon associated particularly with C_3 plants, oxygen is consumed while CO_2 is released from compounds derived directly from intermediates of the CO_2-fixation pathway itself. This subject has been reviewed by Jackson and Volk[28], Black[9] and Zelitch[49].

An oxygen-consuming reaction which occurs readily at least *in vitro*, and which recently has become popular as an explanation of photorespiration, is the ribulose diphosphate oxygenase reaction[33], the products of which are phosphoglycerate and phosphoglycollate. The reaction is catalysed by the same protein (ribulose diphosphate carboxylase) which brings about the initial step of CO_2-fixation, and the product of this carboxylation reaction is phosphoglycerate alone. The oxygenase reaction's mechanism is generally thought to involve oxygen itself[33], though it has been suggested[46] that superoxide is required for oxidation of a sulphydryl group at the active site of the enzyme. No inhibition of the oxygenase reaction by superoxide dismutase has yet been reported, and an 'apparent' superoxide dismutase (which is heat-stable) may even be associated with the active purified enzyme.[39] It should be mentioned that there is by no means general acceptance that ribulose diphosphate oxygenase contributes appreciably to photorespiration. Zelitch[50], for example, attaches importance to a disparity between the pH optima of the carboxylase and oxygenase

reactions, and to the lack of evidence that phosphoglycollate is produced by ^{14}C labelling of intact, photorespiring tissue.

Another process involving oxygen consumption and production of glycollate was proposed by Coombs and Whittingham.[12] Here hydrogen peroxide is generated in a Mehler reaction and leads to production of glycollate from a transketolase intermediate. Kaiser's demonstration[30] that hydrogen peroxide is inhibitory to net CO_2-fixation by intact chloroplasts has already been mentioned. If the Coombs–Whittingham explanation of photorespiration and the 'Warburg effect' (inhibition of photosynthesis by increasing oxygen concentration) is correct, then an increase by hydrogen peroxide of chloroplast glycollate production should occur.

Kirk and Heber[31] have recently published data showing that hydrogen peroxide is inhibitory to chloroplast glycollate production, while addition of catalase brings about a stimulation of glycollate production even without previous addition of hydrogen peroxide to the chloroplast suspension. A number of the observations of Kirk and Heber[31] suggest that glycollate is produced in intact chloroplasts directly from ribulose diphosphate, with the proportion of carbon fixed in glycollate increasing both with oxygen concentration and with pH in the range pH 7.2–8.2. One piece of evidence which in particular seems to rule out a transketolase intermediate as a source of glycollate in intact chloroplasts is the inhibition by the uncoupler FCCP of formation of glycollate from dihydroxyacetone phosphate.[31] This suggests that production of glycollate does not occur at any point between dihydroxyacetone phosphate and the phosphoribulokinase step of the Calvin cycle, but instead occurs after the phosphoribulokinase reaction (the first ATP-requiring step encountered by carbon flowing from dihydroxyacetone phosphate).

The fate of glycollate and the source of photorespiratory CO_2 is also a controversial topic. A contribution of photosynthetic oxygen reduction here may take the form of a requirement for hydrogen peroxide for oxidative decarboxylation of glyoxylate to formate[14, 48], though even if this reaction does participate in photorespiration it is possible that it does so in leaf peroxisomes rather than in chloroplasts themselves.[23] Elstner and Heupel[14] have demonstrated decarboxylation of a number of α-keto acids by chloroplasts in the presence of an autoxidisable electron acceptor, and with NADP (in the presence of ferredoxin) as electron acceptor the decarboxylation commenced as soon as all the NADP present had been reduced. Photorespiration by such a mechanism could be linked to any pseudocyclic phosphorylation which may occur *in vivo*, and so hydrogen peroxide generated in a Mehler reaction may yet be shown to play an important role in photorespiration, even if, as suggested by Kirk and Heber[31] it plays no part in synthesis of glycollate itself.

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